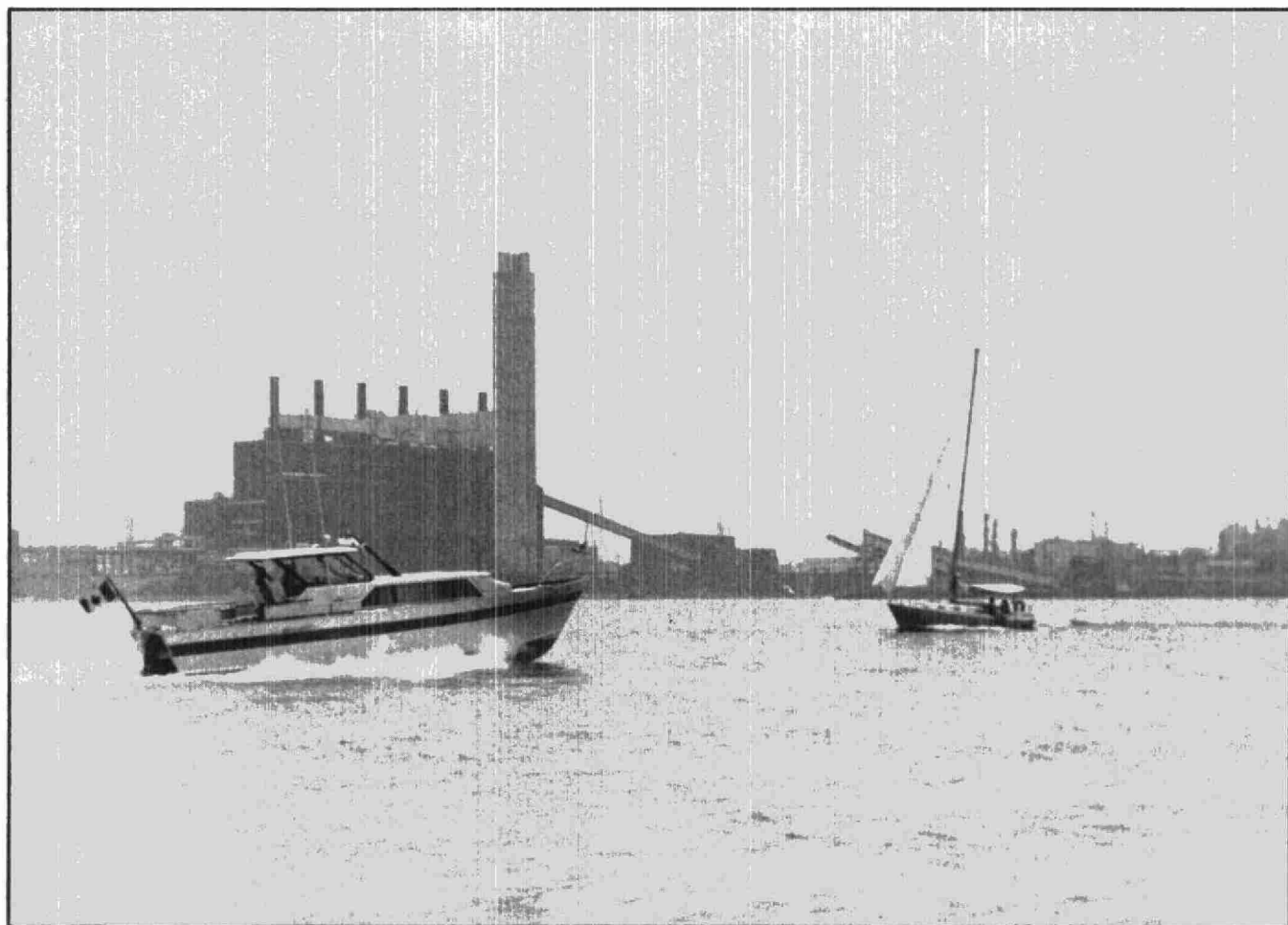


ST. CLAIR RIVER ORGANICS STUDY

BIODEGRADATION OF ORGANIC COMPOUNDS



Ministry
of the
Environment

Hon. Harry C. Parrott, D.D.S.,
Minister

Graham W. S. Scott,
Deputy Minister

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ST. CLAIR RIVER STUDY - EXPLANATORY NOTE
BIODEGRADATION OF ORGANIC COMPOUNDS

The possible breakdown of organic compounds to less toxic substances by natural bacteria living in the water and on the river bottom was investigated as part of the St. Clair River Organics Study.

This was considered essential to estimate the long-term impact of these chemicals in the river environment.

Laboratory testing suggested that numerous chlorinated organic compounds found in industrial discharges are toxic to river bacteria and are therefore unlikely to be subject to biodegradation. Other non-chlorinated compounds, such as ethanol and n-butanol, were shown to be biodegradable. Of 14 compounds tested, five were subject to bacterial breakdown.

The biodegradation study indicates that the elimination of compounds from industrial discharges that are toxic to river bacteria would likely permit the river's "bio-changers" to break down other organic compounds.

ST. CLAIR RIVER ORGANICS STUDY
BIODEGRADATION OF ORGANIC COMPOUNDS

Southwestern Regional Laboratory
Technical Support Section

Ministry of the Environment, London

November, 1980

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FOREWORD

Investigations of organic chemicals in industrial effluents along the St. Clair River commenced in 1975 after the adoption of gas chromatography - mass spectrometry analytical methodologies by the Laboratory Services Branch of the Ministry of the Environment made the detection of low concentrations of such compounds possible. In a preliminary report 'Organic Compounds of Industrial Origin in the St. Clair River' developed as an internal working document in January of 1977, positive identification was made of some 47 organic compounds discharged to the St. Clair River.

The St. Clair River Organics Study Group was formed early in 1977 in response to concerns about the presence and significance of the organic compounds identified, particularly in relation to possible effects on public water supplies downstream and the potential bio-accumulation of such compounds by fish resident in or frequenting this important waterway. Also, it was felt that a comprehensive assessment of water quality conditions and aquatic life was warranted to permit a comparison with unpublished biological data collected in 1968.

Representation on the Study Group included staff from various branches of the Ministry of the Environment and the Special Studies and Services Branch of the Ministry of Labour. Early in its deliberations, the Study Group identified the following goal:

To assess the presence and significance of organic chemical compounds in the St. Clair River system and to establish from this programme recommendations for control measures and further studies that may be required in relation to human health and environmental effects.

In order to accomplish the above goal, the following ten objectives were identified:

1. Identification and quantitation of organic compounds in industrial discharges, St. Clair River water, potable water supplies, bottom sediments and fish.
2. Completion of a literature search on the characteristics and effects of the organic compounds identified. Lethal, sub-lethal and synergistic effects of these compounds on human and aquatic life to be considered.
3. Determination of the significance of chlorinating trace amounts of these organic materials in industrial cooling water discharges, sewage plant effluents and in potable water supplies.
4. Determination of the types and concentrations of compounds causing fish toxicity and fish tainting problems.

5. Estimation of the dissolved organics concentration at any point in the river based on effluent quality and flow measurements.
6. Completion of benthic macroinvertebrate, aquatic plant, sediment and water quality studies on the river in terms of biological and common chemical indicators. Results to be compared to survey work carried out in 1968-69. Assessments of mercury levels in sediments to be completed.
7. Determination of the potential carcinogenicity of organic compounds through quick-screening microbiological tests for mutagenesis.
8. Establishment of whether biodegradation or alteration of organic compounds occur through bacterial action in sediments.
9. Development of recommendations clarifying levels to which contaminants must be reduced, as a basis for necessary treatment and effluent controls.
10. Clarification of the need for additional research to establish potential dangers to human health or the natural environment where this information is lacking.

Work on the component studies related to these objectives progressed throughout 1977 and 1978 and into 1979. As a result of these efforts the following reports will be published during 1979 and 1980, to form an integrated series covering the objectives defined previously.

- (1) Identification and Quantitation of Organic Compounds.
- (2) Fish Toxicity and Tainting Evaluations for Selected Industrial Effluents.
- (3) Waste Dispersion.
- (4) Biological Surveys 1968 and 1977.
- (5) The Detection of Mutagenic Activity; Screening of Twenty-three Compounds of Industrial Origin.
- (6) Biodegradation of Organic Compounds.

Wherever possible, each of these reports will present conclusions and advance recommendations to achieve an improvement of water quality in the St. Clair River system or to clarify additional research requirements in regard to potential human health or environmental effects.

It is anticipated that subsequent reports dealing with fish toxicity and tainting and the potential carcinogenicity of industrial organic compounds in relation to the St. Clair River will be issued by the Ministry, since these particular components will be the subject of further study by the Limnology and Toxicity Section of the Water Resources Branch and the Microbiology Section of the Laboratory Services Branch, respectively.

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This project was conducted by the microbiology staff of the Southwestern Regional Laboratory under the direction and supervision of Mr. G. Palmateer^a. Chemicals used in the study were supplied by Dr. O. Meresz^b. The biology unit of the Water Resources Assessment section provided the field sampling under the direction of Mr. J. D. Westwood^c.

- a. Microbiologist, Southwestern Regional Laboratory,
Ontario Ministry of the Environment, London, Ontario.
- b. Manager, Organic Trace Contaminants Section, Ontario
Ministry of the Environment, Toronto, Ontario.
- c. Biologist's Assistant, Ontario Ministry of the Environ-
ment, London, Ontario.

SUMMARY

The experimental data produced from this study, demonstrate that numerous organic compounds found in the St. Clair River are unlikely to undergo a significant degree of biodegradation. Hence, most potentially toxic organic compounds discharged to the river will remain in the water and sediment with the exception of those compounds subject to degradation, volatilization and photodecomposition.

The degradation that was shown to occur suggests that short carbon chain compounds ($C_2 - C_6$) such as alcohols and other aliphatic hydrocarbons would readily be oxidized to less toxic metabolites. However, the chlorinated, insoluble compounds studied are generally non-biodegradable. The presence of toxic organic compounds in sediments of the St. Clair River serves to inhibit beneficial degradation processes.

It has been postulated that many of the organic and aromatic hydrocarbons with specific gravities greater than one would be deposited on the bottom of the river. This process occurs as a result of hydrocarbons being absorbed by particles such as phytoplankton, zooplankton and detritus that settle to the bottom. Since this is a continuous process, the above-mentioned toxic chemicals would accumulate to various depths in the sediment (Lee et al., 1978). Evidence of this theory of sedimentation was observed during the sampling of the river sediment and based on toxicity and degradation studies included in this study, these anthropogenic compounds could exert long-lasting effects on the river benthos.

RECOMMENDATIONS

1. Similar assessments of biodegradability would be useful for additional organic compounds identified by the Microbiology Section of the Laboratory Services Branch as potentially significant in the St. Clair River System.
2. Suggested future studies using more advanced techniques such as $^{14}\text{CO}_2$ evolution from [^{14}C] compounds and chromatographic - mass spectrometric techniques would serve to not only identify the metabolites of bacterial decomposition but would demonstrate the kinetics of degradation. The rate of degradation of particular organic compounds would further our understanding of their potential significance by providing information regarding their chemical half-life in the environment.

INTRODUCTION

Many synthetic organic chemicals produced by petrochemical industries may eventually find their way into aquatic environments. The major sources of chemical industrial pollution have been identified as the following: cooling water discharges, process effluents and waste processing effluents (activated sludge; oxidation ponds) (Gloyna and Ford, 1970).

As a result of biological and/or chemical degradation mechanisms, toxic substances may be altered such that the resultant compounds are either less toxic than the originals or, in some cases, may exhibit an even greater toxicity to the surrounding environment. Microbial transformations play perhaps the single most important role in the detoxification of organic chemicals. This report describes the evaluation of chemical-bacterial toxicity and the potential for biodegradation of selected organic chemicals found in sediment and water of the St. Clair River near Sarnia.

Of the 70,000 toxic chemicals listed in the new Toxic Substances Control Act of the U.S. Environmental Protection Agency, little is known of their biodegradability (Alexander, 1979). By definition, biodegradability is the minimum modification of a compound necessary to change the identity of the compound (WPCF, 1967). It can also be defined as the alteration of a chemical which is susceptible to an attack by the enzyme apparatus acquired by microbes during the course of their evolution (Dagley, 1972b). The efficiency of the biodegradation process for a specific substance depends upon a variety of factors such as temperature, pH, aeration, nutrient capacity, molecular structure, concentration and solubility, as well as its toxicity to the microbial population in its proximity (Abrams, et. al., 1975).

Hydrophobic compounds (i.e. chemicals of limited aqueous solubility) and dissolved organic solvents from industrial discharges interact upon entering the receiving waters. A partitioning occurs between hydrophobic compounds, dissolved organic solvents, water and particulate matter suspended in the water. This process serves to bring the hydrophobic compounds into a closer association with the sediment bacteria and in turn, stimulates biological utilization of the chemicals (Sayler and Colwell, 1976, Walker and Colwell, 1976).

Several studies have identified a variety of organic chemicals found in the St. Clair River and in industrial effluents discharging to the river. (Gloyna and Ford, 1970, Duholke and Meresz, 1980). Some of these chemicals have been classified as refractory while others have been considered to be readily biodegradable. However, no single classification system can apply in all circumstances since so many factors affect the adaptation or the acclimation of the microorganisms in the receiving environment (Abrams et. al., 1975). If a particular microbial population receives continuous quantities of a specific chemical previously classified as recalcitrant, degradation of that chemical may occur as a result of an acclimation to it. Of the microbial populations found in the water and sediment of the St. Clair River, bacteria are probably the most adaptable owing to their ability to reproduce rapidly and to genetically manufacture those enzymes necessary to degrade a particular organic compound. In addition, the genetic information initially responsible for this adaptation may be passed on to other bacterial genera by the transference of chromosomal material called plasmids. This process increases the number of bacteria capable of degrading specific organic compounds (Chakrabarty, 1978).

One of the most important factors affecting microbial adaptation, and subsequently biodegradation, is the initial toxicity of the chemical compound. If the concentration of the chemical is high enough, and the chemical exhibits toxicity to the microbial population present in the environment, then detoxification of the chemical may be impossible as a result of either lethal or sub-lethal injury to the microbial community (Colwell and Sayler, 1978).

In this study, natural environmental conditions of the St. Clair River and the condition of the sediment below specific industrial discharges were considered in designing chemical toxicity evaluation and biodegradation experiments. The chemicals chosen for the study had limited solubilities. The majority possessed a specific gravity greater than one that would increase the likelihood of finding them in the river sediment (Table 1).

FIELD SAMPLING PROCEDURES

Samples of bottom sediments were obtained using a Ponar dredge at all of the locations indicated on the accompanying map (Figure 1) and specifically identified in Table 2. Sediment samples for bacteriological analysis were collected in sterile wide mouth glass jars and were stored at approximately 6°C until they were returned to the laboratory for analysis.

Where it was considered that the sediment had remained undisturbed after sampling, pH readings were taken in triplicate using a Radiometer glass combination electrode

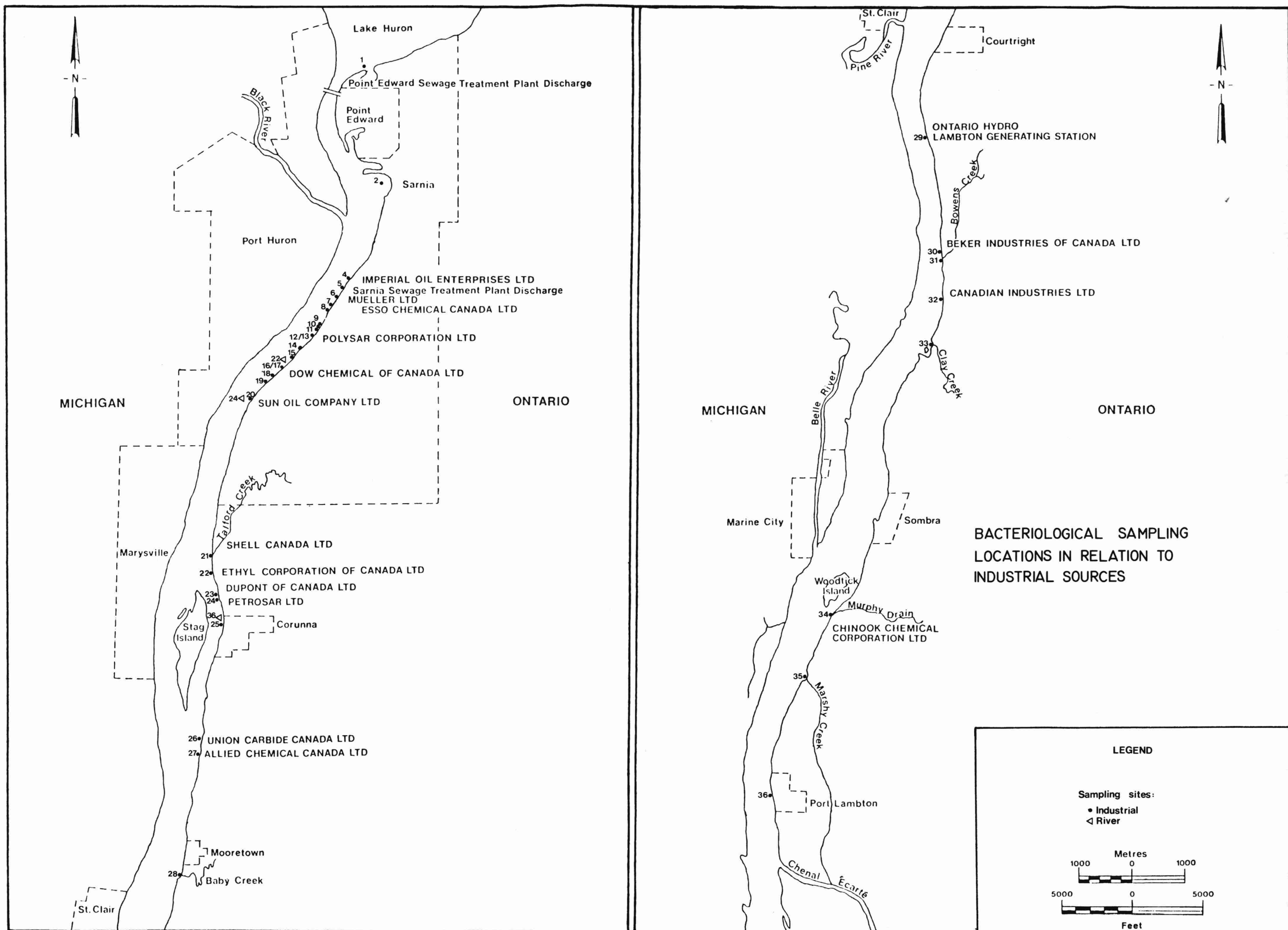


Figure 1

and redox potential readings were taken with an Orion platinum combination electrode, in conjunction with a portable Radiometer Model 29 pH meter. Each electrode was housed in a polyethylene chamber which allowed the electrodes to penetrate the sediment to a depth of 8.0 mm. At the same time, the sediment was saturated with distilled water. The redox potential was allowed to stabilize before the actual redox readings were recorded. The apparatus is shown in Plate 1.

The temperature of the water was also determined and noted. Water temperature, sediment pH and redox potentials are summarized in Table 3.

Biotransformation reactions would be unlikely inhibited at the pH values detected in the sediment; however, the water temperature at several industrial site locations was considerably higher than the river stations; thereby affecting the rate of bacterial metabolism. Redox (reduction-oxidation) potentials in the sediment at those locations of less than 150 mv indicate that conditions are not favourable for the oxidation of organic chemicals with the exception where sulfate-reducing bacteria are capable of hydrocarbon oxidation. However, the redox potential in the sediment would have to be less than 30 mv for this slow degradation process to occur and thereby, be significant.

Table 1. Specific gravities and aqueous solubilities
of selected organic chemicals used in this study

<u>CHEMICAL NAME</u>	<u>SPECIFIC GRAVITY</u>	<u>AQUEOUS SOLUBILITY</u>
tetrachloroethane	1.5866	3,000
hexachloro-1,3-butadiene	1.6820	i
tetrachloroethylene	1.6311	400
trichloroethylene	1.4649	1,100
styrene		i
benzene	0.8787	700
xylene (mixture of o-,m-p-forms)	0.8600*	175
2-chloroethyl alcohol	1.2003	∞
1-phenylethyl alcohol	1.0129	N.A.
n-butanol	0.8098	73,000
ethanol	0.7980	

i - insoluble

∞ - soluble in all proportions in water

N.A. - not available

from CRC Handbook of Chemistry and Physics 52nd edition

Table 2. Site numbers and location of sampling
points along the St. Clair River

<u>SITE NUMBER</u>	<u>LOCATION</u>
1 Control	1 mile into Lake Huron
I.S. 21	Mouth of Telford Creek
I.S. 32	Below C.I.L.
I.S. 7	Esso - below No. 9 separator
I.S. 14	Below Dow
I.S. 16/17	Dow 3rd Street sewer / DOEO
I.S. 18	Dow 4th Street sewer
I.S. 22	End of Polysar property
I.S. 24	Dow (between Dow and Sunoco)
I.S. 36	Below Petrosar

I.S. = Industrial site

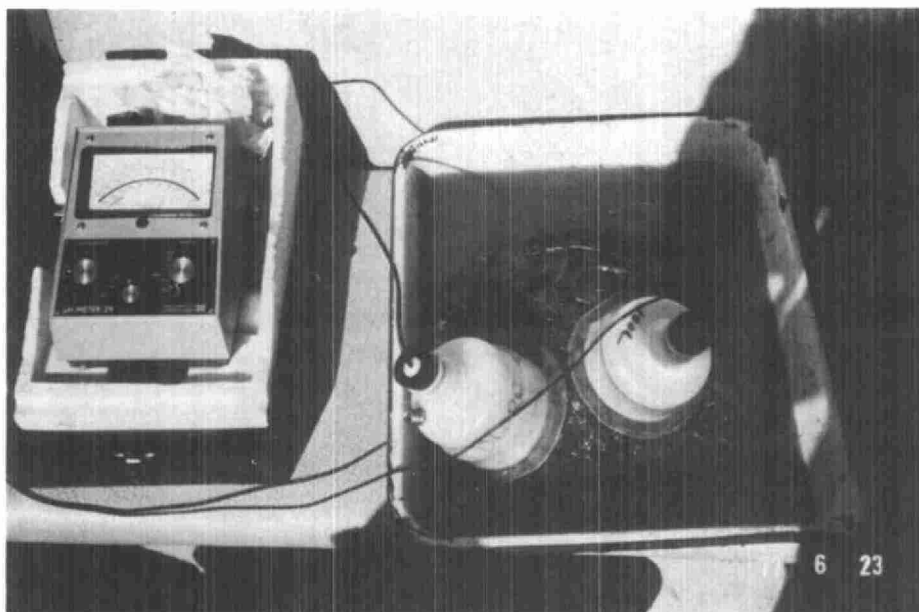


Plate 1. pH meter with pH and E_h electrodes set in sediment sample of the St. Clair River.

Table 3. Temperature (surface water) pH and redox potential of sediments from industrial site locations and selected river sites.

Industrial Site Station Numbers	Surface Water Temperature in oC	pH	Eh (Redox in mv)
7	27	7.6	+307.7
8	20	7.5	+319.7
9	20	7.9	+320.7
11	21	6.9	+ 44.0
12	21	7.0	- 22.3
14	25	7.1	+ 64.3
15	20	8.5	+ 74.3
16	20	7.6	- 82.0
19	20	7.5	+ 54.0
20	20	7.6	+ 93.2
21	35	6.9	+128.3
22	20	6.9	+107.3
23	20	7.2	+134.0
24	20	7.4	+151.5
25	20	7.2	+104.0
26	21	7.1	+124.0
27	21	7.2	+ 50.6
28	25	6.7	+ 63.5
29	30	7.6	+293.0
30	21	7.3	+241.5
33	24	7.3	+ 27.3
34	24	7.2	+ 97.3
35	22	7.0	+157.3
36	20	ND	ND
River Site			
Station Numbers			
22	14	7.5	+151.0
24	10	ND	ND
36	17	ND	+ 90.7

METHOD OF ENUMERATION OF BACTERIAL DENSITIES

Sediment from each sample was weighed aseptically and 20.0 g were transferred into individual sterile blender jars. After the addition of 180 ml of phosphate buffer at pH 7.2, the samples were blended for one minute. Ten ml from each jar were transferred separately by pipette into 90-ml phosphate buffer dilution blanks. Serial 10-fold dilutions were then prepared aseptically and 0.1 ml aliquots of specific dilutions were plated onto CPS, casein-peptone-starch agar employing the spot plate technique, in duplicate. (Ontario Ministry of the Environment, 1977) One set of these plates was incubated aerobically while the second set was incubated anaerobically. Both sets of plates were incubated at 20°C for 7 days.

Anaerobic jars with anaerobic generators supplied by Baltimore Biological Laboratories were used for the anaerobic incubations.

The concentrations of aerobic and anaerobic heterotrophic bacteria as determined at sampling sites are listed in Table 4. As can be observed from Table 4, the levels of aerobic heterotrophic bacteria range from 10^5 to 10^7 per g of sediment depending upon the station location. The concentrations of anaerobic heterotrophic are somewhat lower ranging from 10^4 to 10^6 per g of sediment. Upon ascertaining this data, the results were used to design biodegradation experiments using bacterial levels which are representative of those in St. Clair River sediment.

Table 4. Concentrations of aerobic and anaerobic heterotrophic bacteria in sediment of the St. Clair River

Industrial Sites Station Numbers	Aerobic Hetero- trophic Plate Count (per g of sediment)	Anaerobic Heterotrophic Plate Count (per g of sediment)
4	11×10^6	80×10^4
5	1.1×10^6	78×10^4
7	89×10^6	460×10^4
8	27×10^6	24×10^4
9	51×10^6	470×10^4
10	70×10^6	200×10^4
11	750×10^6	180×10^4
12	--	1000×10^4
14	40×10^6	600×10^4
15	15×10^6	220×10^4
16	35×10^6	150×10^4
18	27×10^6	100×10^4
19	2.3×10^6	29×10^4
20	55×10^6	17×10^4
21	80×10^6	12.5×10^4
22	102×10^6	110×10^4
23	52×10^6	75×10^4
24	5.2×10^6	33×10^4
25	0.55×10^6	1×10^4
26	3.8×10^6	15×10^4
27	8.3×10^6	47×10^4
28	4.4×10^6	35×10^4
29	17×10^6	2×10^4
30	770×10^6	3.5×10^4
31	4.9×10^6	7.5×10^4
32	28×10^6	17×10^4
33	6.3×10^6	26×10^4
34	2.5×10^6	33×10^4
35	61×10^6	160×10^4
36	59×10^6	80×10^4
Geometric Mean:	20.4×10^6	52.0×10^4

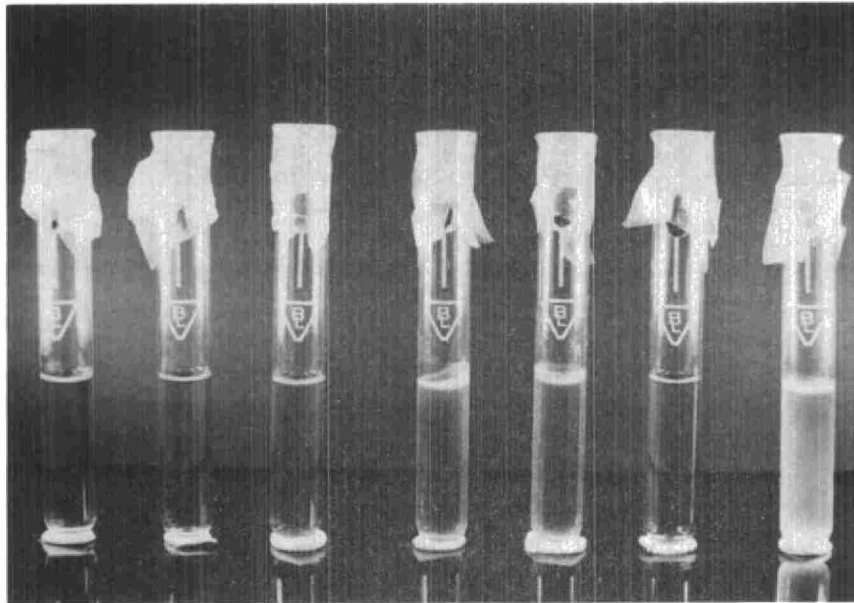


Plate 2. Cuvettes containing varying degrees of turbidity as a result of growth of sediment bacteria.

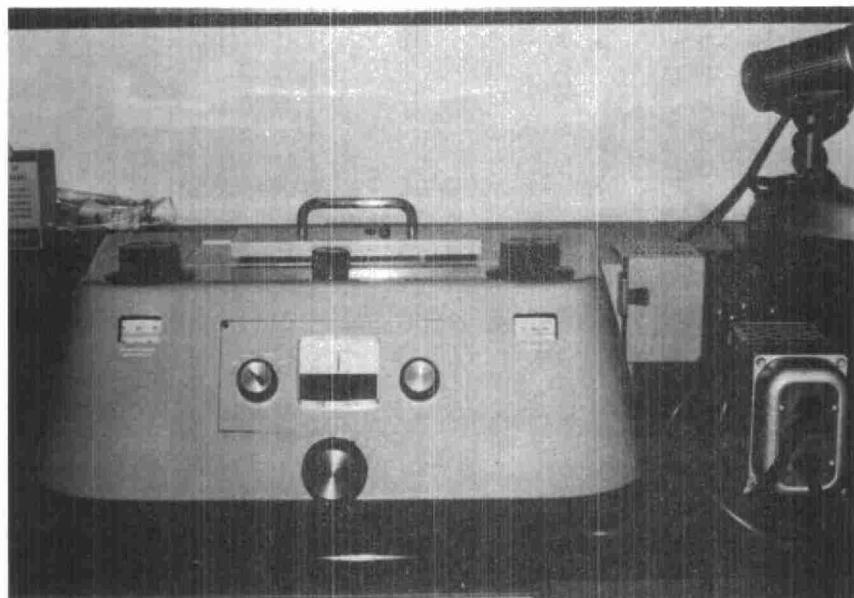


Plate 3. Pye-Unicam spectrophotometer used to measure the amount of turbidity in cuvettes.

TOXICITY TESTING OF ORGANIC COMPOUNDS ASSESSED
USING TURBIDIMETRIC MEASUREMENTS

An experimental method was developed to assess the growth response of a mixed culture of bacteria recovered from sediments of the St. Clair River when exposed to selected organic chemicals. The chemicals studied had varying aqueous solubilities and specific gravities as shown in Table 1. Many of the chemicals have been described as being toxic to bacteria by some authors, (Smith, 1974). Other scientists have demonstrated that similar chemicals act as nutrient sources for microorganisms (Abrams, et al, 1975; Markovetz, 1971). In this study, therefore, it was deemed necessary to examine the potential toxicity of specific compounds to the microbial flora of the river sediment.

Method

Owing to the specific gravity and low aqueous solubility of many of the compounds being examined, a simple toxicity test was designed. A mixed inoculum of bacteria initially recovered by a direct plating technique of sediment dilutions was exposed to the chemicals listed below.

- 1) 1-phenylethanol alcohol
- 2) Iso-butanol alcohol
- 3) hexachloro-1,3-butadiene
- 4) ethanol
- 5) tetrachloroethylene
- 6) benzyl alcohol
- 7) trichloroethylene
- 8) n-butanol
- 9) benzene
- 10) 2-chloroethanol

In previous studies these chemicals were categorized according to their biodegradability and ranged from refractory to easily decomposed (Table 5). Five volumes of each chemical, specifically 2.5, 5.0, 10.0, 15.0 and 20.0 ul, were injected using a microlitre syringe into individual cuvettes containing 4.0 ul of trypticase soy broth. Each volume of every chemical was duplicated in another matched cuvette. A control for each chemical was set up by combining 20.0 ul of the chemical with 4.0 ml of the trypticase soy broth. A biological control was also employed which contained 0.01 ml of inoculum and 4.0 ml of the trypticase soy broth.

After the addition of the chemical, each tube other than the chemical control tubes was inoculated with the mixed culture using a quantitative loop of 0.01 ml (see culture preparation on page 22). Each tube was then immediately capped with parafilm and incubated statically at 20°C. After an equilibrium period of approximately six hours, turbidity readings were taken using a Pye-Unicam SP 600, Series 2 spectrophotometer (Plate 2). The instrument was zeroed using the chemical control as a blank at a wavelength setting of 520 m . A second set of readings was taken at approximately twelve hours. These readings were in an optimal absorbance range using this type of spectrophotometer (Plate 3).

Results and Discussion

The results of the toxicity assay are presented in the figures, 2 through 5.

As a result of this type of toxicity test, the growth response of bacteria recovered from the sediment to five aliquots (0.0 to 20.0 ul) of individual chemicals was evaluated.

It can be observed from Figure 2 that a slight toxicity was demonstrated with n-butanol at the 15.0 and 20.0 ul quantities. At 2.5 and 5.0 ul the growth response indicated a complete absence of toxicity and possible n-butanol utilization as the turbidity was higher in each instance than in the biological control sample without the chemical.

In the case of iso-butanol (Figure 2), a similar response to n-butanol was observed indicating that the branched form of the molecule had little stereochemical effect on its toxicity. This result was in contrast to the findings of other authors (Schaeffer et al., 1979).

The highly chlorinated compound, hexachloro-1, 3-butadiene (Figure 2), demonstrated no toxicity at any of the five quantities of chemical. Unlike the butyl alcohols described above, the chemical appeared to be nontoxic at any volume. Previously, the Environmental Protection Agency had categorized this compound as refractory (Howard et al., 1975).

The growth response to ethanol (Figure 3) indicated a slight toxicity as all turbidity readings were less than the biological control. The chlorinated form of

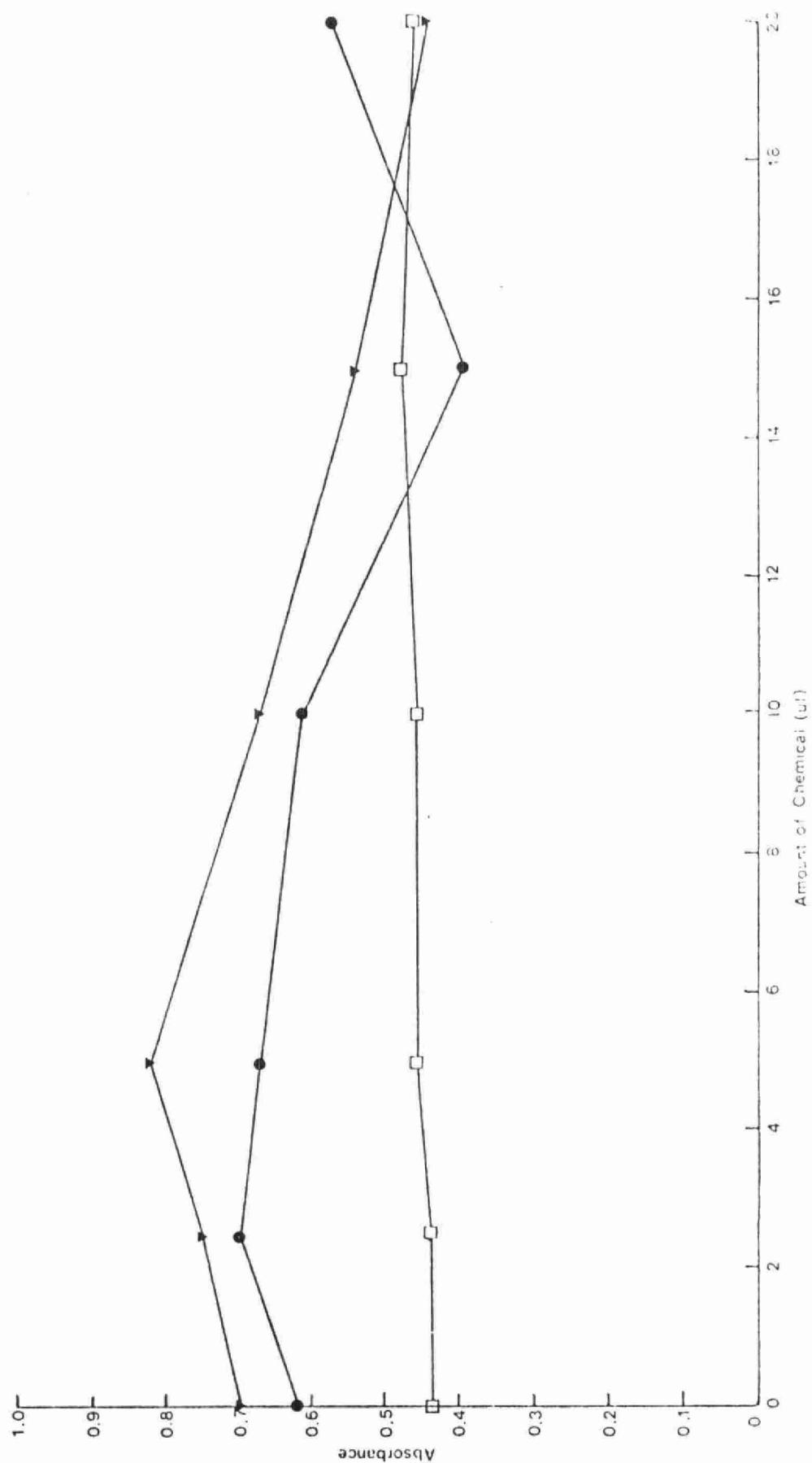


Figure 2. Growth Response to n-Butanol (▼), iso-Butanol (●), Hexachloro-1,3-butadiene (□).

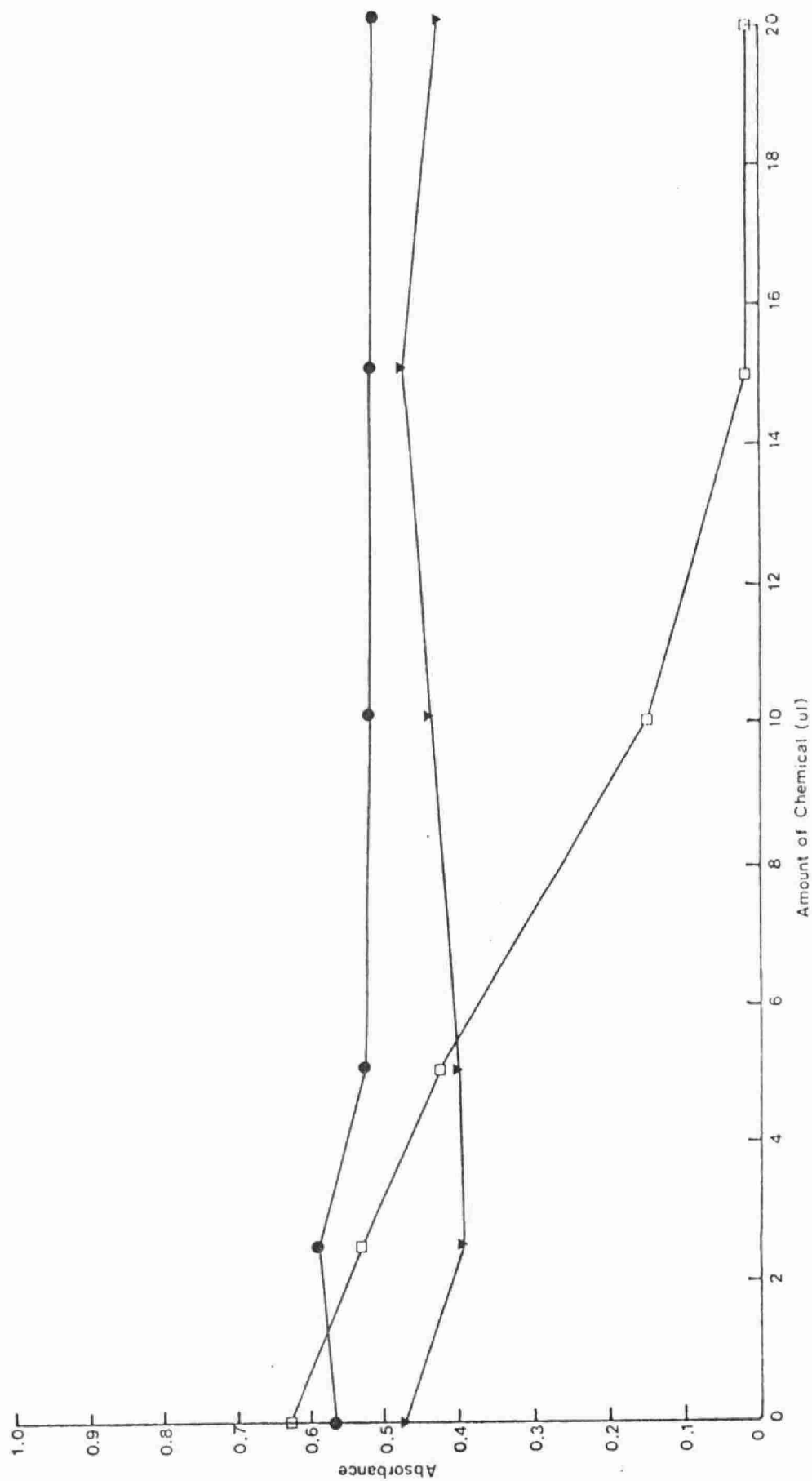


Figure 3. Growth Response to Ethanol (▼), 2-Chloroethanol (●), 1-Phenylethanol (□).

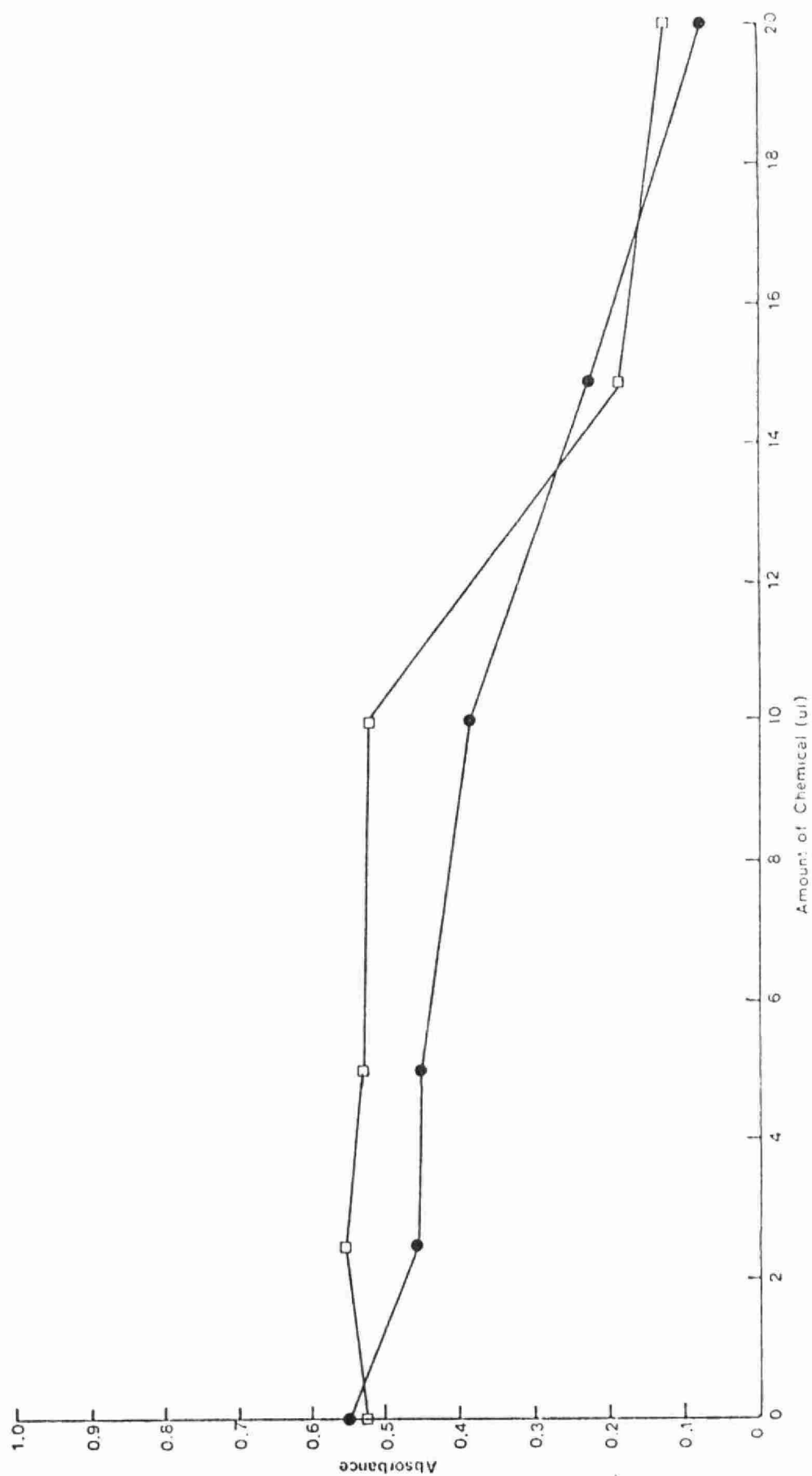


Figure 4. Growth Response to Benzene (●), Benzyl alcohol (□).

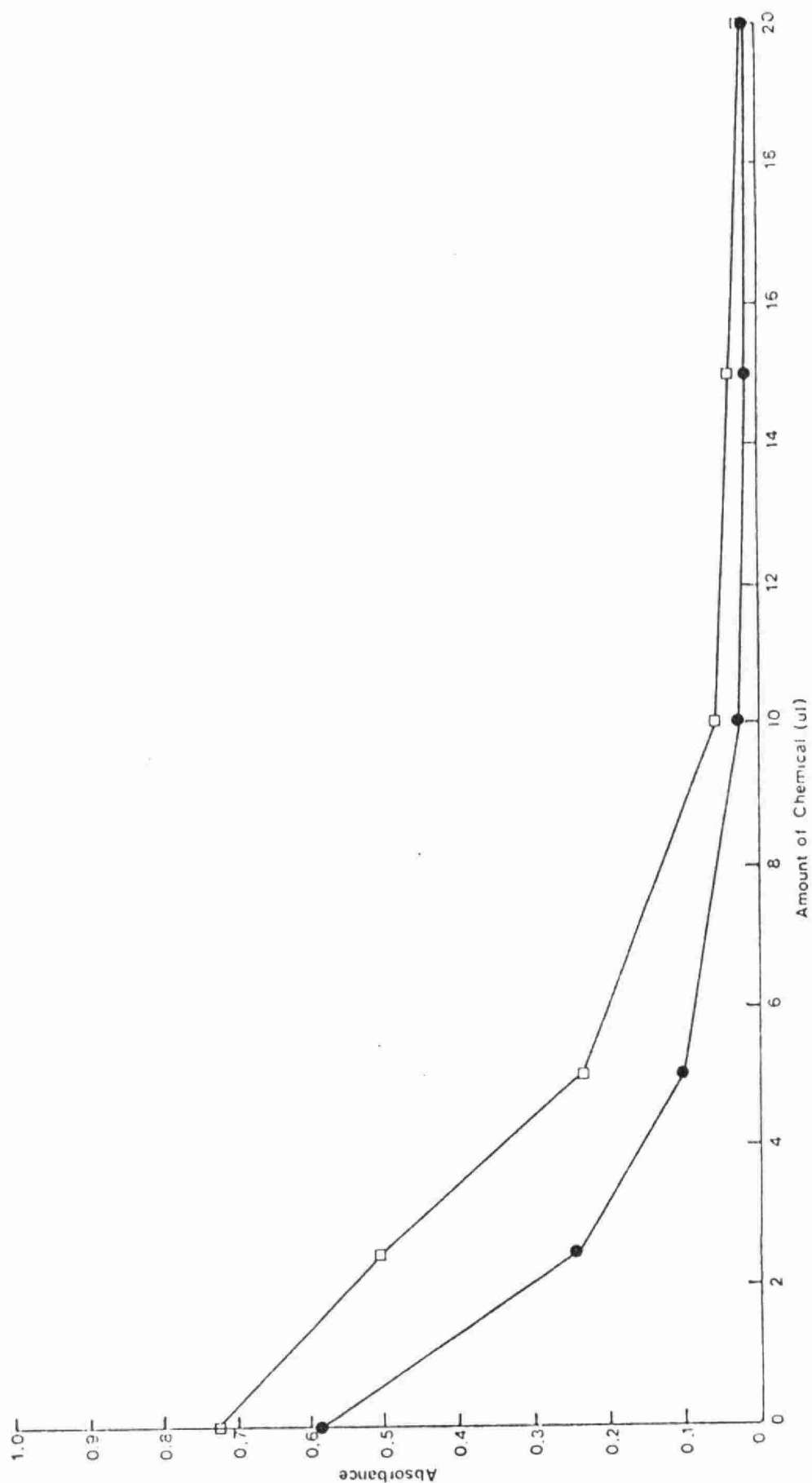


Figure 5. Growth Response to Tetrachloroethylene (●), Trichloroethylene (□).

the molecule, 2-chloroethanol, as shown in Figure 3, demonstrated a similar response; however, the 1-phenylethanol compound (Figure 3) exhibited a strong toxic effect as the bacterial growth was extremely limited at the 15.0 and 20.0 ul values. Based on the comparison of the growth response to the other quantities of the chemical used, the relationship appeared to be inversely proportional.

In Figure 4, benzene demonstrated a definite toxicity to the sediment bacteria at the 15.0 and 20.0 ul amounts; however, the toxic nature of the chemical decreased considerably at lower concentrations. The toxicity observed for benzyl alcohol (Figure 4), was unusual in that the growth response at 15.0 and 20.0 ul amounts was approximately one-third that of the control value. At 2.5, 5.0 and 10.0 ul quantities, the toxicity was absent as the absorbance values were similar to that of the control value.

As can be observed in Figure 5, tetrachloroethylene demonstrated a significant toxicity to sediment bacteria as growth was barely detectable at 10.0, 15.0 and 20.0 ul. At 2.5 ul, the growth response was only 42.4% of the biological control sample without the chemical.

Trichloroethylene (Figure 5) also appeared to exhibit a strong toxicity to the sediment bacteria. This response was only slightly less than that of tetrachloroethylene. The growth response in the 2.5 ul concentration was 69.2% of the biological control value. The result suggests the manner in which the toxic qualities of a molecule may change with the difference of only one chlorine atom.

BIODEGRADATION OF ORGANIC COMPOUNDS

INOCULUM PREPARATION

Sediment samples were collected from the river in sterile wide-mouth jars using a Ponar dredge. The sample locations, as shown in Figure 1, were directly below industrial outfalls. The sediment samples were returned to the laboratory after completion of the sampling and were refrigerated at 4°C. Sediment analyses were conducted within a 24 hour period.

Twenty-gram samples of sediment were weighed out and transferred to a sterile blender jar containing 180 ml of phosphate buffer. The sediment slurry was then blended for one minute. Ten ml of this mixture were pipetted from the blender jar into phosphate-buffered dilution blanks. After a series of 10-fold dilutions were prepared, plates containing CPS agar were inoculated using the spread plate technique. Following incubation at 20°C for seven days, the plates were examined for growth. Typical colonies from each plate were then picked and inoculated into a trypticase soy broth tube, labeled as per the location. The industrial sites chosen for the experiment are displayed in Table 2. Hence a set of tubes containing mixed inocula representing each station was produced. The tubes were incubated at 20°C for four days, thereby providing time for the more slowly growing organisms to reach higher concentrations.

EXPERIMENT 1

This experiment was designed to determine the potential for biodegradation under laboratory conditions of a series of organic compounds previously identified in the river.

Method

Particular chemicals suggested by the literature to possess varying degrees of biodegradability were chosen. These chemicals are listed in the following table.

Table 5. Biodegradability of selected organic compounds
(Abrams et al. 1975)

Refractory Compounds:

hexachloro-1,3-butadiene

Very Difficult to Degrade Compounds:

tetrachloroethylene

trichlorethylene

Difficult to Degrade Compounds:

xylene

benzene

Compounds Degraded Without Much Difficulty:

benzyl alcohol

1 - phenylethanol

2 - chloroethanol

Easily Degraded Compounds:

n-butanol

ethanol

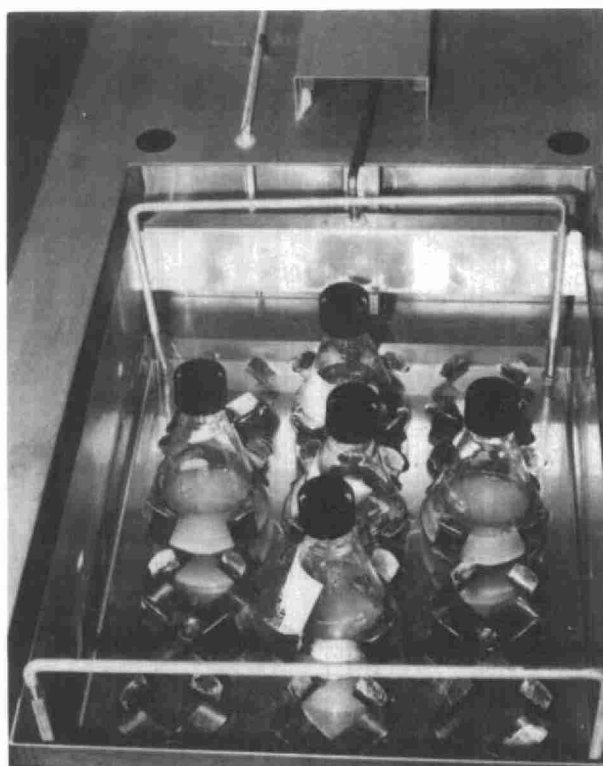


Plate 4. Reciprocating shaking water bath used to agitate nutrients and inoculum in flasks.



Plate 5. Phase contrast microscope used to observe bacteria present in growth medium.

Each chemical (0.5 ml) was individually added to 100 ml of basal mineral broth and placed in a 250-ml Erlenmeyer flask with a screw cap. The composition of the basal mineral broth, which contained essential inorganic nutrients for the organisms, was as follows: ammonium sulfate, 2g; calcium chloride dihydrate, 0.1g; magnesium sulfate septahydrate, 0.1g; potassium dihydrogen phosphate, 0.08g; sterile purified water, 1000 ml; pH 7.2.

Each flask was inoculated with a 24-hour mixed population in trypticase soy broth, at concentrations similar to those found in the river sediment. Two control flasks were also prepared. A flask labeled Biological Control contained the basal mineral broth and inoculum while a second flask labeled Chemical Control contained basal mineral broth and ethanol. Ethanol was chosen as the control chemical because it was considered readily biodegradable. All flasks were incubated at 25°C for ten days in a reciprocating shaking water bath (Plate 4).

Results and Discussion

Degradation of the chemical as the sole carbon source in each flask was detected by the development of turbidity, an indication of the growth of bacteria and/or fungi. The amount of biodegradation in each flask was judged with respect to the control flasks by comparing the amount of turbidity. Each flask was also observed using a Nikon SK-T phase contrast microscope to confirm the growth response (Plate 5). Trypticase soy agar plates were streaked from all experimental and control flasks. The growth response to each chemical is displayed in Table 6.

Table 6. Growth response as a result of degradation
of various organic chemicals

<u>CHEMICAL</u>	<u>GROWTH RESPONSE</u>
2-chloroethanol	+++
isopropanol	+++
hexachloro-1,3-butadiene	++
benzene	-
xylene	+
ethanol	+++
n-butanol	++++
1-phenylethanol	-
tetrachloroethane	-
trichloroethylene	-
Chemical Control	-
Biological Control	++

+ - indicates amount of turbidity

- - indicates absence of turbidity

Microscopically, bacterial growth was observed which indicated that the higher densities of organisms, expressed as +++ or greater in the respective flasks, was a result of the utilization of the specific hydrocarbon. Growth in the Biological Control flask was most likely due to a carbon residual from the trypticase soy broth introduced into the flask during the inoculation procedure, thereby providing an additional carbon source. Growth on trypticase soy agar plates confirmed these microscopic observations as an assortment of bacterial colonies was observed.

Since the growth in the presence of this group of hydrocarbons was not enumerated, the observations regarding

the above chemicals provided a basis for a similar experiment in which the cells were washed, thereby freeing them of the organic growth medium, trypticase soy broth. Cell counts were also conducted to further substantiate the biodegradability of the chemicals studied.

It appeared from the data in Table 6 that degradation of ethanol, 2-chloroethanol, isopropanol and n-butanol occurred. Since the growth in hexachloro-1, 3-butadiene was observed to be identical to the biological control, degradation did not appear to have occurred; however, the chemical did not appear to be toxic to the organisms in the river sediment. Benzene, 1-phenylethanol, tetrachloroethane and trichloroethylene appeared to be toxic to the sediment bacteria.

EXPERIMENT 2

This experiment was designed to demonstrate the potential for biodegradation of particular organic compounds by a variety of bacteria recovered from St. Clair River sediment.

Method

Inoculum preparation was similar to the previous biodegradation experiment; however, an additional cell washing step was included in an attempt to decrease the amount of organic matter added to the flasks during inoculation.

Trypticase soy broth tube cultures from each industrial site were incubated for three days at 20°C. Inoculum from each tube (excluding the control tubes) was transferred aseptically into 100 ml of trypticase soy broth contained in a 250 ml Erlenmeyer flask. The flask was incubated for an additional three days at 20°C on an oscillating gyrosaker at 150 r.p.m. (Plate 5).



Plate 6. Oscillating shaker used in culturing bacteria growing in response to organic chemicals.

As mentioned previously, a cell washing step was instituted in this experiment. Following the second three-day incubation period, 5.0 ml of the mixed culture was removed from the flask and dispensed into a 15.0 ml polypropylene-capped centrifuge tube. The cells were spun at 1840 xg for 15 minutes. After centrifugation, the supernatant was carefully withdrawn from the test tube using a 5-ml sterile Pasteur pipette. Five ml of basal mineral broth were added to the tube containing the pellet of bacteria and were mixed by means of a Fisher Super-Mixer. The cells were washed an additional two times. The final suspension of cells was then used for further inoculations.

The chemicals chosen for the biodegradation evaluation were as follows: trichloroethylene, carbon tetrachloride, tetrachloroethylene, benzene, xylene, 2-chloroethanol, 1-phenylethanol, ethanol, styrene (plus anti-oxidant).

One hundred microlitres of each of the above chemicals was injected individually using a Hamilton 100- μ l gas tight syringe into 100 ml of basal mineral broth contained in a 250-ml Erlenmeyer screw cap flask. All 10 flasks were then inoculated with 0.01 ml of the washed cell suspension. A biological control flask containing inoculum and basal mineral broth was prepared. The flask containing ethanol and inoculum was considered as a positive control. The flasks were left shaking for 15 minutes on an oscillating gyrosaker (Plate 6) at 150 rpm. before the cells in each flask were enumerated. This was accomplished by use of the spot plate technique and CPS agar. The concentration of the cells in each of the flasks was determined as mentioned in the previous biodegradation experiment at 7 days and at 15 days.

Results and Discussion

The levels of growth of the bacteria as shown in Table 7 indicate the growth response to the carbon source available for metabolism.

It was observed from the results of the seven-day incubation of n-butanol that an obvious anomaly had occurred. Therefore, experimental procedures above were repeated for n-butanol as well as for a control flask. The resulting data are distinguished from the first run by the superscript "a" in Table 7.

By comparing the bacterial concentrations in the flasks containing the various chemicals to the bacterial levels in the control flask after both incubation periods, it appears that n-butanol was the only compound degraded. However, based on the degree of turbidity produced it was observed that significant growth occurred in the flask containing ethanol. It appears that some of the organisms adapted to degrading the ethanol did not grow on CPS agar, thereby giving an underestimation of the bacterial densities and extent of degradation.

The differences in growth responses in the two degradation experiments may have resulted from residual trypticase soy broth being introduced into the growth flask via inoculation in the first experiment, whereas in the second experiment the bacterial cells were washed free of any external carbon source before inoculation. Residual carbon from the trypticase soy broth probably resulted in cometabolism between the various chemicals and the external organic carbon source.

TABLE 7. Concentrations of bacteria in response to a variety of carbon sources

<u>Chemical Name</u>	<u>Numbers of bacteria per ml of growth medium</u>		
	<u>Time in Days</u>		
	0	7	15
trichloroethylene	6.0×10^4	0.0	0.0
tetrachloromethane	2.0×10^4	10.5×10^5	19.0×10^4
tetrachloroethylene	20.5×10^4	0.0	180×10^4
2-chloroethanol	35.0×10^4	15.5×10^5	48.5×10^4
1-phenylethanol	33.5×10^4	165×10^5	24.0×10^4
n-butanol ^a	2.0×10^4	6200×10^5	18.0×10^4
benzene	13.0×10^4	33.5×10^5	380×10^4
xylene	11.0×10^4	15.5×10^5	110×10^4
ethanol	8.5×10^4	42.0×10^5	75.5×10^4
styrene	57.3×10^4	0.0	80×10^4
control	31.5×10^4	300×10^5	1800×10^4
control ^a	4.4×10^4	450×10^5	ND

a repeated experiment with n-butanol and appropriate control

BIODEGRADATION OF HEXACHLORO-1,3-BUTADIENE
IN BASAL MINERAL BROTH AND RIVER SEDIMENT

This experiment was designed to assess the potential for biodegradation of hexachloro-1,3-butadiene in the St. Clair River sediment as compared to its potential degradation in basal mineral broth.

Method

A 250-ml. screw cap Erlenmeyer flask containing 100 ml of basal mineral broth, 50 g of sediment and 1 ml of hexachloro-1,3-butadiene, labeled Experimental Flask A, was inoculated with a mixed culture from Station 22. The culture was prepared as described on page 22. The inoculum was composed of a variety of bacteria recovered from the river sediment below the Polysar outfall (Station 22). A similar flask, labeled Experimental Flask B, was prepared containing the above components with the exception of the sediment. The sediment had been previously prepared by air-drying it in a fume hood for 7 days and then sterilizing it by autoclaving the sediment at 121°C for one hour. Two controls were used. Chemical Control A contained 100 ml of basal mineral broth, 50 g of sterile sediment and 1.0 ml of hexachloro-1,3-butadiene. The Biological Control B contained 100 ml of the broth and 0.01 ml of the inoculum.

All flasks were incubated at 25°C in a reciprocating shaking waterbath. After a brief equilibration period, all flasks were analyzed in duplicate to determine the initial concentration of inoculum using the spot plate technique with CPS agar. A similar analysis was conducted after 20 days of incubation.

Results and Discussion

The results at the commencement of the experiment and after the 20-day incubation period are presented in Table 8.

Table 8. Growth response of bacteria to
hexachloro-1,3-butadiene
Concentration of bacteria per ml

<u>FLASK</u>	<u>0 TIME</u>	<u>20 DAYS</u>
Chemical Control A	0	0
Chemical Control B	0	0
Biological Control A	38,500	1,300,000
Biological Control B	36,500	1,200,000
Experimental A	38,000	4,275,000
Experimental B	39,000	17,500

It can be observed from Table 8 that although there was a similar increase in cell numbers in both the biological controls, with and without sediment, the change in the experimental A and B flasks was significantly different. In experimental flask A, containing sediment, chemical and bacteria, an increase in the bacterial concentration occurred with respect to either biological control. In contrast, growth in experimental flask B, which did not contain the sediment, indicated that hexachloro-1,3-butadiene concentration of 1.0 ml of the chemical in 100 ml of the basal mineral broth was apparently toxic to the bacteria. The organisms in the flask containing sediment may have been supplied some essential nutrient(s) that encouraged the production of specific enzymes which ultimately resulted in the degradation of the chemical. Without the presence of the sediment, however, the metabolism of hexachloro-1,3-butadiene did not seem to occur and eventual die-off of the sediment bacteria ensued.

BIODEGRADATION OF STYRENE IN BASAL MINERAL BROTH
AND RIVER SEDIMENT

The following experiment was designed to determine the potential for the biodegradation of styrene by bacteria found in the sediment of the St. Clair River. This experiment was conducted primarily in view of research involving the degradation of styrene in the environment (Sielicki et al., 1978, Leibman, 1975).

Method

The 48 hour mixed inoculum used in this experiment was recovered from the sediment of the St. Clair River below the Polysar outfall, as styrene had been previously recovered in the effluent.

The styrene used contained an antioxidant t-butylpyrocatechol at 10-15 ppm.

A 250-ml Erlenmeyer screw cap flask, labeled A, containing 100ml of basal mineral broth, 1.0 ml of styrene, and 50 g of sterile river sediment was inoculated with 0.01 ml of a mixed bacterial culture. The sterile river sediment was prepared as in the previous experiment. A similar control flask, labeled CS, containing 100 ml of basal mineral broth, 1.0 ml of styrene and 50 g of sterile river sediment, was also employed. A biological control, labeled BC containing 0.01 ml of inoculum, 50 g of sterile sediment in 100 ml of basal mineral broth was also prepared. A second experimental flask, labeled B, contained 100 ml of basal mineral broth, 1.0 ml of styrene and 0.01 ml of inoculum. The chemical control flask, labeled C, contained only 100 ml of basal mineral broth and 1.0 ml of styrene. All flasks were incubated in the dark to prevent photo-decomposition of the styrene and were shaken in a waterbath at 25°C as shown in plate 4.

Results and Discussion

The initial inoculum concentration in the flasks and the sterility of specific controls was determined by plating duplicate sample aliquots on CPS agar. The plates were then incubated at 20°C for 7 days. The concentrations of bacteria determined are displayed in Table 9.

Table 9. Growth response of bacteria to styrene

<u>Numbers of bacteria per 100 ml</u>		
<u>Flask</u>	<u>0 Time</u>	<u>7 Days</u>
Experimental A	147,500	0
Sediment Control CS	0	0
Experimental B	147,500	0
Control C	0	0
Biological Control BC	147,500	670,000

It appeared from the data that styrene appeared to be extremely toxic to the test organisms at a concentration of 1% (vol/vol). As a result of its specific gravity of 0.907, styrene would also tend to be found on the surface of the water, thereby limiting the potential for the sediment bacteria to adapt to this compound. For this reason, the sediment bacteria were also unable to detoxify styrene which appears to have caused a drastic reduction of the bacterial population.

DISCUSSION

It has been demonstrated that the interaction of selected organic compounds of industrial origin with bacteria in river sediment can occur. Such factors as pH, temperature, dissolved oxygen, availability of essential inorganic nutrients as well as chemical concentration, aqueous solubility, sorptibility to solids (clay and organic matter) and molecular structure all have an effect on whether a compound is recalcitrant or biodegradable. After measuring a number of these parameters in the St. Clair River, experiments were designed to examine the toxicity of those chemicals previously identified in the effluents of petrochemical industries as well as in water and river sediment.

When a chemical is toxic to microorganisms, biodegradation of that chemical is unlikely to occur; however, if the chemical is non-toxic, it may be subject to microbial decomposition. Hence, after the toxicity of various chemicals to bacteria from the St. Clair River sediment was established, the potential for biodegradation of the chemicals was evaluated. Since experimental variables employed were as similar to in situ conditions as possible, the evaluation of both toxicity and degradation was considered to be representative.

The toxicity testing demonstrated that the aliphatic alcohols, ethanol and n-butanol, which are more soluble than the aromatic, benzyl alcohol, were much less toxic to the sediment bacteria than the aromatic compounds.

An increase in the number of chlorine atoms on a molecule usually increases its toxicity and thereby decreases the potential for degradation (Tabak et al., 1965). Tetrachloro- and trichloroethylene did demonstrate significant toxicity to the test bacteria. However, hexachloro-1,3-butadiene did not exhibit toxicity to the sediment bacteria and was found to be a potentially degradable compound.

Both the aromatic compounds tested, benzene and benzyl alcohol, although varying in aqueous solubility as well as being non-chlorinated, exhibited only a slight toxicity to sediment organisms. Hence both compounds appeared to be potentially biodegradable by bacteria found in the St. Clair River sediment.

Based on the results of the toxicity experiments, specific compounds established to be present in the St. Clair River were selected for biodegradation experiments. The various biodegradation experiments demonstrated that the more polar, soluble compounds such as ethanol and n-butanol were biodegradable at temperatures, pH values and bacterial concentrations similar to river conditions, while the relatively non-polar, (aqueous) insoluble compounds appeared to be non-biodegradable, with the exception of hexachloro-1,3-butadiene. The experiment using a growth medium composed of sterile river sediment and inorganic nutrients provided an environment in which the sediment bacteria, possibly through cometabolism, were able to degrade this chlorinated compound. This was not observed, however, with styrene which was toxic to the sediment organisms at the concentration used.

The lack of degradation of the compounds such as tetra- and trichloroethylene, tetrachloroethane may be due to their degree of chlorination. Benzene and xylene may have been difficult to decompose due to their molecular structure

and lack of aqueous solubility. Other factors such as metal concentrations which have been shown to inhibit bacterial metabolism (Walker and Colwell, 1976) and a lack of an oxidizing environment, as evidenced by the oxidation-reduction potential in the sediment, may have also had a significant effect in slowing the decomposition rate. Mercury concentrations, which were detected as high as 58 mg/kg of sediment, have been demonstrated to be toxic to bacterial enzymes (Vanderpost and Corke, 1975). In addition, the oxidation-reduction potentials below +150 millivolts indicate that conditions in the sediment were not ideal for the oxidation processes to occur (Hewitt, 1950, Hargrave, 1972).

Another factor which might have adversely affected microbial degradation involved the distribution of the organics in the sediment. Compounds such as tetrachloroethylene and trichloroethylene, which were found to be toxic to the sediment bacteria, were observed at numerous sites along the Canadian side of the river (Duholke and Meresz, 1977). Decomposition of other non-toxic and potentially degradable compounds may therefore have been significantly inhibited.

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